Semi-Synthesis to Examine the Role of Phosphorylation of Histone H3 at T118

A Senior Honors Thesis

Presented in Partial Fulfillment of the Requirements for graduation *with research distinction in Biochemistry* in the undergraduate colleges of The Ohio State University

by
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Chapter 1: Introduction

The eukaryotic genome is organized into chromatin. The base unit of chomatin is the nucleosome, consisting of 147 base pairs of DNA wrapped around an octamer core of two of each histone protein, H2A, H2B, H3, and H4 [1]. Nucleosomes must be dynamic structures in order to regulate access to DNA by transcription and repair, yet all DNA must be tightly packaged within the nucleus. Modification of chromatin structure is thus one mechanism of regulating biological processes such as DNA transcription and repair [2]. Chromatin remodeling complexes are known to alter higher order chromatin structure and affect the transcription of genes [3]; post-translational modifications also have the potential to dramatically alter chromatin structure [4].

Post-translational modifications have been well-characterized in the tail region of histone proteins, leading to the "histone code" hypothesis, that post-translational modifications of histone proteins provide cues to gene expression [5]. In the tail, the protein structure is less rigid and does not contribute to the quaternary structure of the nucleosome [6]. Recently, over 30 histone modifications within the core of the nucleosome have been detected by mass spectrometry in mammalian histones [7]. Many of these modifications lie on the lateral surface, thus directly interacting with DNA in the nucleosome [6]. The phosphate backbone of nucleosomal DNA makes specific contacts with the histone octamer; therefore, modifications at this surface may be able to alter the structure of the DNA on the nucleosome, or the nucleosome as a whole[6].

Threonine 118 of histone H3 (H3-T118) is located at the dyad region of the nucleosome at the histone-DNA interface, directly adjacent to the DNA phosphate backbone [Figure 1], [6], and has been demonstrated to be phosphorylated in calf thymus histones [7]. H3-T118 is one

location known as a Sin mutant. When mutated to Ile, there is no need for chromatin-remodeling complexes to activate genes in yeast [8]. Furthermore, the mutation leads to increased sliding and depositioning of the nucleosome *in vitro* [9]. Also, mutation of the residue to Ala or Glu is lethal to yeast[10]. However, crystal structures of several different H3-T118 mutants reveal only minimal sidechain rearrangements in nucleosomes [11]. Nevertheless, mutants are not an exact replicate of a modification. T118E has been used to mimic the effect of phosphorylation at T118 by introducing a negative charge and somewhat increased steric bulk. It does not, however, have the same spatial arrangement and steric bulk or charge as a phosphothreonine. To determine the precise impact of phosphorylation, we will introduce a phosphothreonine site-specifically at H3-T118 [Figure 2].

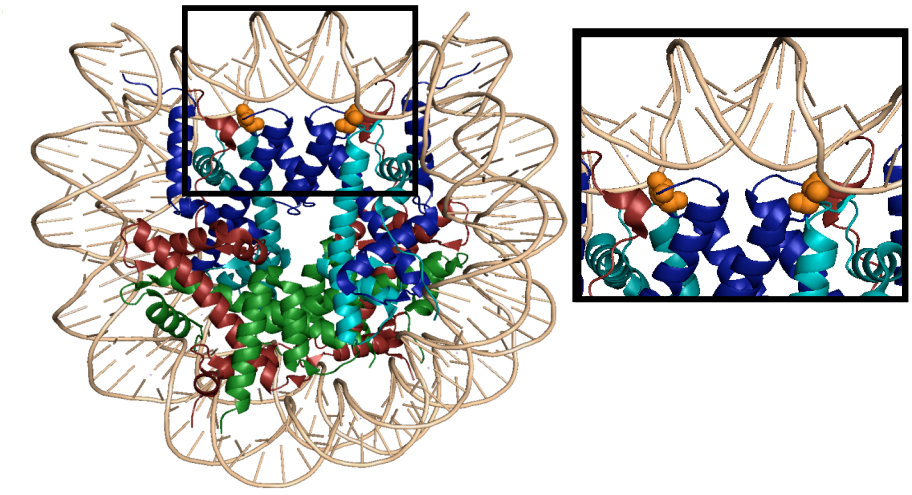


Figure 1: Crystal structure of the nucleosome [6]. T118 is highlighted in orange.

Expressed protein ligation to form H3-pT118 allows for a native protein with T118 specifically phosphorylated [12]. Solid phase peptide synthesis of a peptide containing H3 residues 110-135, including pT118 will be ligated at a native Cys site (C110) to a recombinant

thioester. The resultant ligation product will have a native peptide bond at the ligation site, and preserve the H3 wild-type sequence.

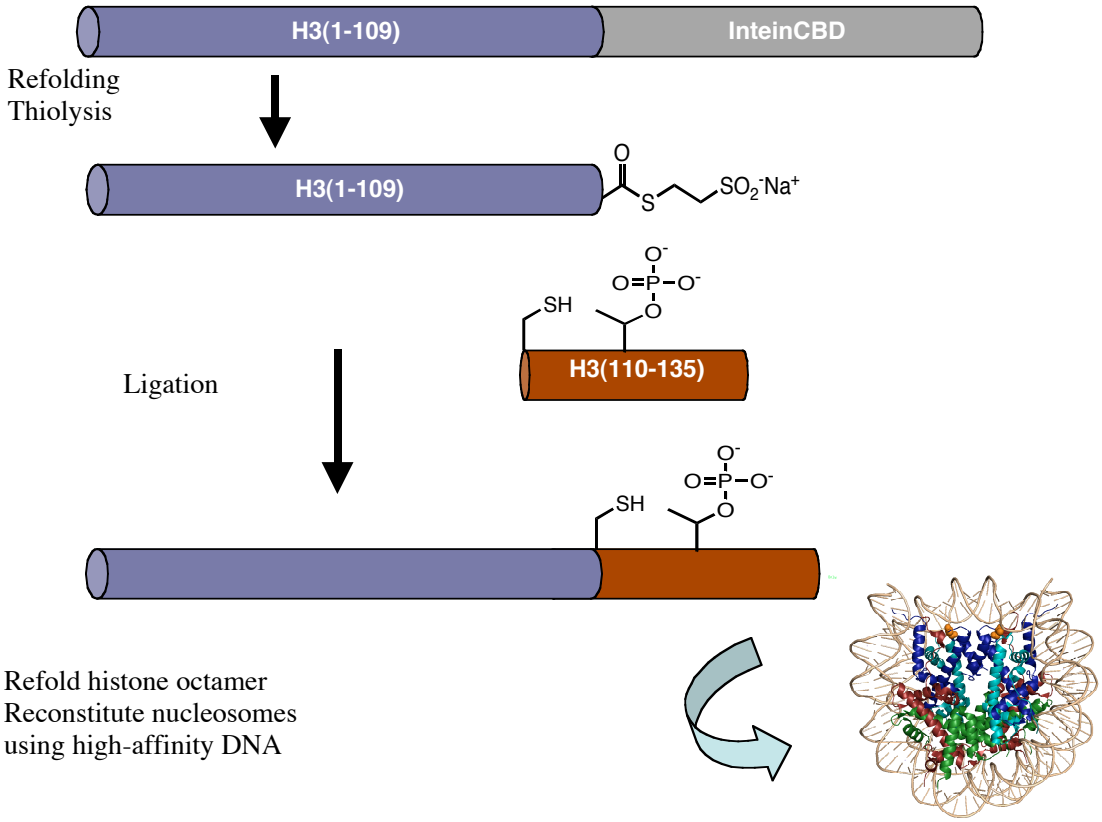


Figure 2: Synthetic Scheme. H3(1-109)SR is cleaved from the intein-CBD, and ligated to synthetic H3(110-135)-pT118. The protein, along with H2A, H2B, and H4, was refolded into the histone octamer, and reconstituted into nucleosomes with labeled DNA.

Chapter 2: Synthesis of H3-pT118, Octamer, and Nucleosomes

I: Synthesis of H3(110-135)-pT118

Semi-synthetic H3-pT118 was generated via ligation of recombinant H3(1-109) thioester to a synthetic H3(110-135)-pT118 peptide. H3(110-135)-pT118 was synthesized on Fmoc-Ala-Wang resin on a 0.125 mmol scale using standard Fmoc synthetic protocols with HBTU as a coupling reagent. To ensure complete coupling occurred, ninhydrin tests were performed after coupling each residue, and capping with acetic anhydride and/or double coupling proceeded as needed. The N-terminal cysteine was incorporated as a thiazolidine (Thz) group to prevent racemization during synthesis [13].

H3-pT118(110-135):

Thz-A-I-H-A-K-R-V-pT-I-M-P-K-D-I-Q-L-A-R-R-I-R-G-E-R-A

Conditions for the cleavage of the peptide from resin were tested using the standard TFA cleavage cocktail, as well as Reagent K [14]. While the first cocktail yielded about 33% product via RP-HPLC and MALDI-TOF mass spectrometry analysis [Figure 3a], Reagent K yielded about 67% product, proving much more efficient at deprotecting the phosphorylated threonine [Figure 3b]. A crude yield of 43.8% was achieved overall.

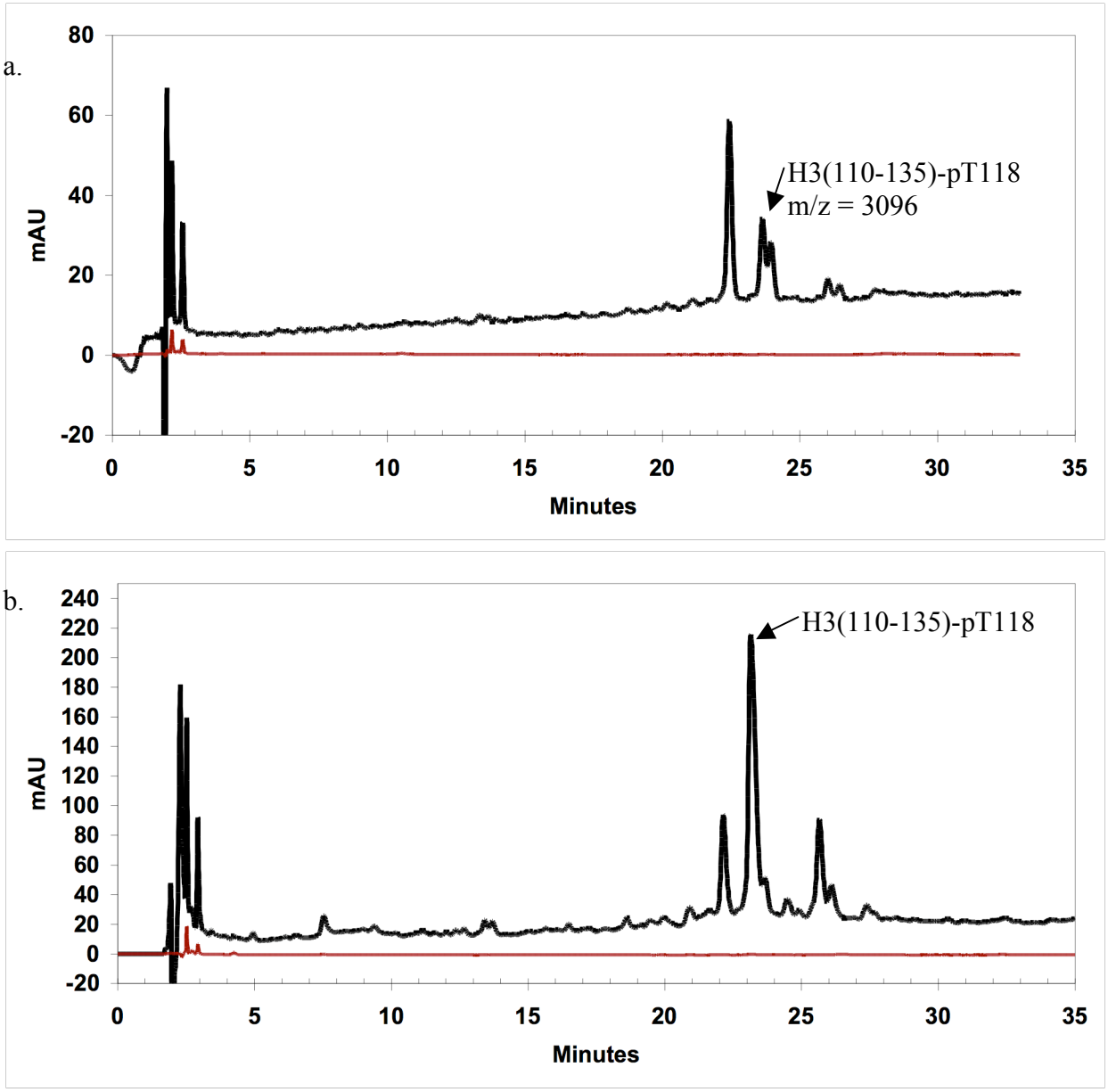


Figure 3: 2 Cleavage Cocktails- 13.5-27% acetonitrile RP-HPLC

a. Initial cleavage after 4 hours- benzylated species is about 67% of total
b. Reagent K cleavage after 2 hours- benzylated species is about 33% of total

RP-HPLC and MALDI-TOF mass spectrometry analysis of the peptide revealed the presence of 3 major species: the desired peptide, the desired peptide plus an extra valine, and the desired peptide with the protecting group still on pT118 (benzylated) [Figure 4a]. Some disulfided peptide dimer that was also observed in the crude; addition of TCEP reduced the species. The extra valine species is likely to have occurred due to the previous amino acid, phosphothreonine, binding piperidine during the deprotection step, or due to poor flow washing after deprotection. This would have allowed for the valine to couple twice, as the presence of piperidine could deprotect the peptide after valine was coupled. This was prevented in the second synthesis of the peptide by adding an extra wash with 10% DIEA after deprotection of the peptide after pT118 [Figure 4b]. This effectively prevented the addition of an extra valine, and none of this species was observed. Crude yields for the second synthesis were 28.6%.

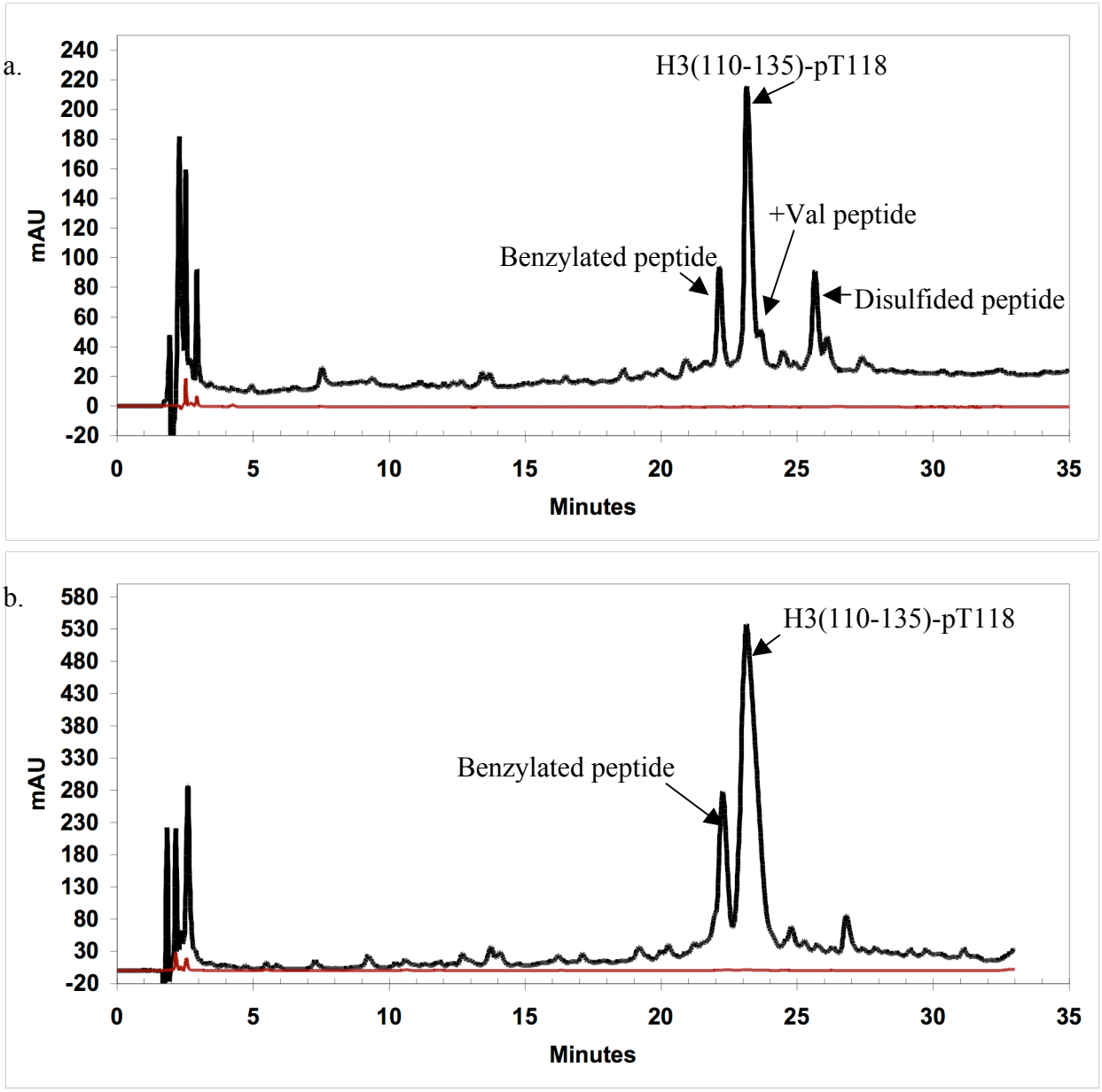


Figure 4: Synthesis with and without extra DIEA step

a. RP-HPLC 13.5-27% acetonitrile, 30 minute gradient of initial synthesis
b. RP-HPLC 13.5-27% acetonitrile, 30 minute gradient of second synthesis in which DIEA wash was utilized

The N-terminus was protected with a thiazolidine group during synthesis in order to prevent racemization of cysteine, which is likely to occur under basic conditions during Fmoc SPPS [13]. After cleavage, the N-terminal thiazolidine group was converted into Cysteine by reaction with 0.4M methoxylamine in 30% acetonitrile at pH 4 [Figure 5]. Reaction completion was confirmed by RP-HPLC and MALDI-TOF analysis.

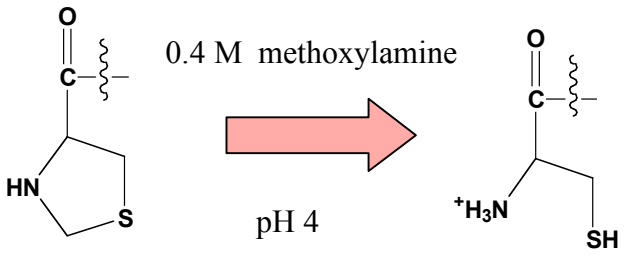


Figure 5: Deprotection Scheme for the N-terminal Cysteine

Large scale purification of H3(110-135)-pT118 was carried out using a C18 Grace Vidac column on a 13.5-27% acetonitrile gradient over 50 minutes. Individual fractions were taken and analyzed via analytical scale RP-HPLC [Figure 6a] and MALDI-TOF [Figure 6b] to determine purity, and pooled. Pure yields were 3.1% for the initial synthesis, and 16.0% for the second synthesis.

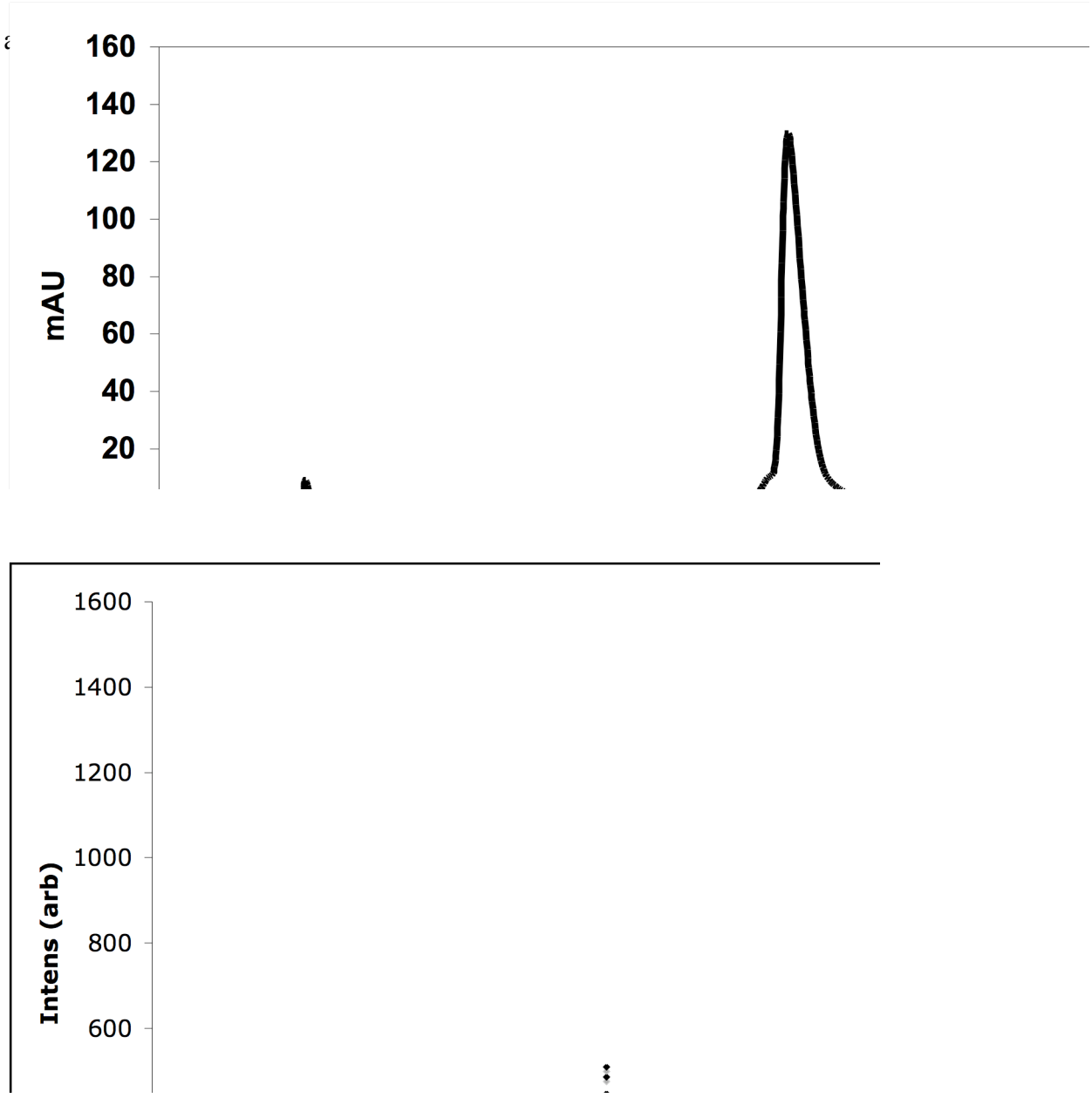


Figure 6: Prep-scale purification of H3-pT118(110-135)

a. Pure pool on a 19.5-25.2% acetonitrile 15 minute gradient
b. Mass spectrometry of pure pool

II: Synthesis of H3(1-109)SR

The vector containing H3(1-109)-intein-CBD had been previously transformed into BL21 cells by Annick Edon and a glycerol stock of these cells was used for these thioester preparations. Overnight cultures of cells from the glycerol stocks were grown. Expression of the protein was induced by the addition of IPTG, and cells were allowed to grow for 2.5 hours. The cell bodies were harvested by centrifugation, resuspended and lysed using a French press. Protein accumulated in the inclusion bodies, so it was purified from the pellet by soaking the lysate pellet in DMSO in order to dissolve the protein into solution. The protein was purified over an anion exchange column, and then over a gel filtration column. Note that the intein-CBD moiety is not utilized in purification, as the CBD does not aid purification of histones. Finally, the thioester was cleaved from the intein-CBD by MESNA. Cleavage from the intein-CBD realiably proceeded to 50% or greater [Figure 7]. The cleavage mixture was then concentrated to >1 mg/mL H3(1-109)SR. Typically, yields of about 6 mg per 3 L of culture were obtained.

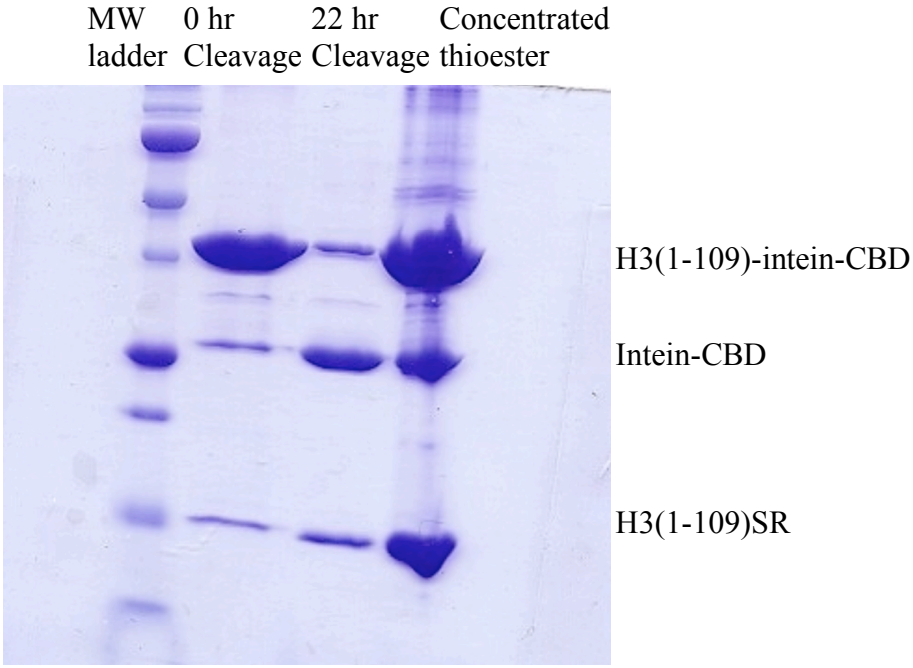


Figure 7: Cleavage gel for H3(1-109)SR. Cleavage proceeded to about 65% in this preparation

III: Expressed protein ligation to form H3-pT118

Ligation of the peptide to the recombinant thioester was achieved by adding peptide directly to the concentrated thioester mixture and adding additional MESNA [Figure 8]. TCEP was added to the system in order to prevent disulfide formation from impeding reaction progress. Ligation reliably proceeded to 50% or greater [Figure 9]. Excess peptide was removed by dialysis.

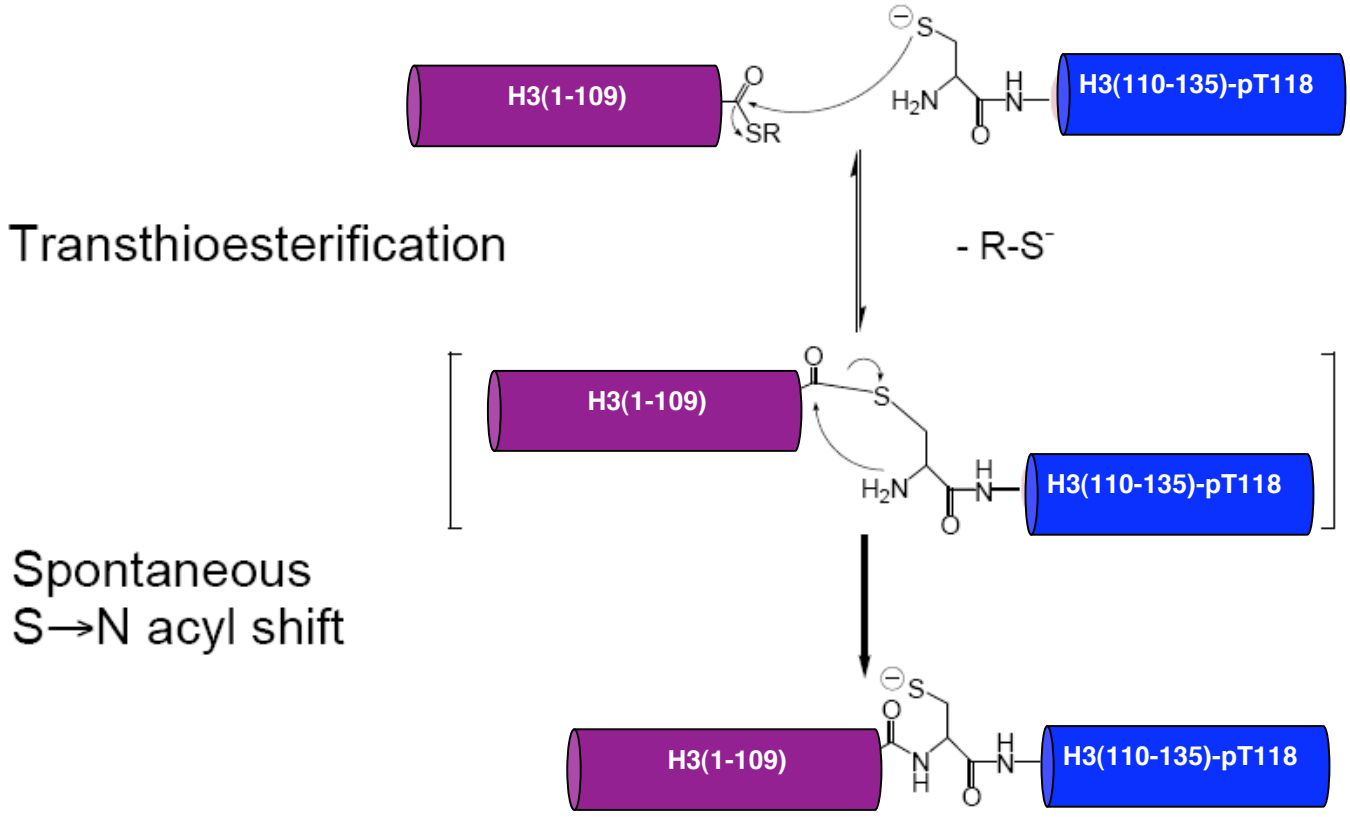


Figure 8: Native chemical ligation [12]

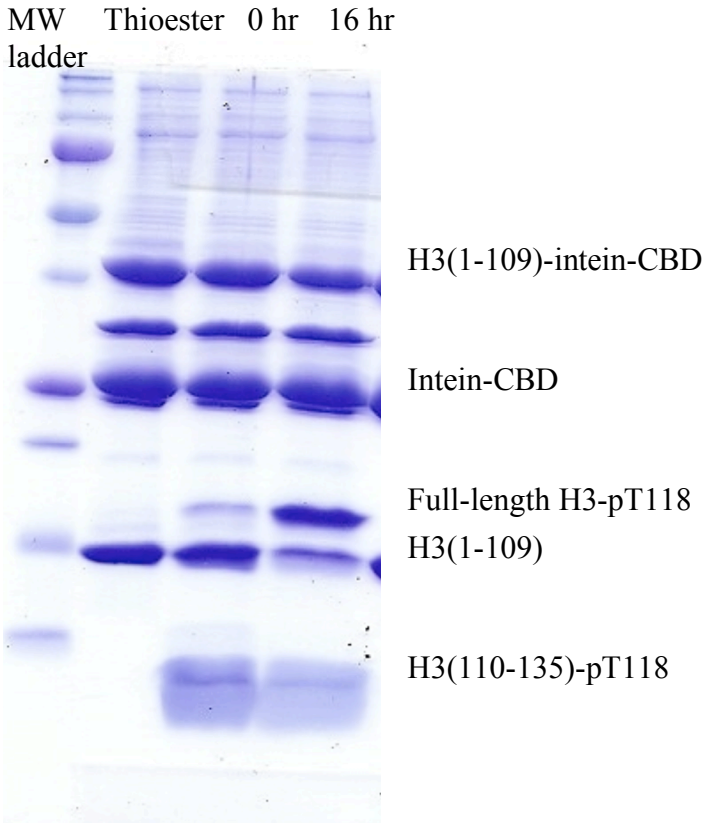
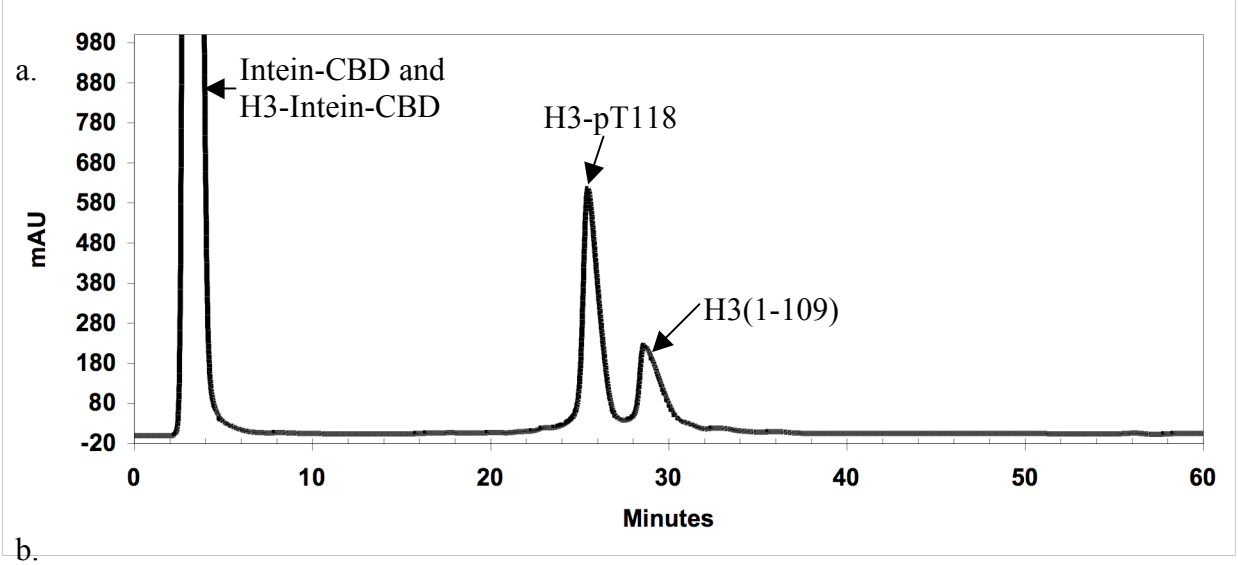


Figure 9: 15% SDS-PAGE of H3-pT118 ligation. Ligation proceeded to ~70% in this preparation.

The full-length H3-pT118 was purified away from the intein-CBD and thioester by ion exchange chromatography under denaturing conditions [15],[Figure 10a]. Complete separation from the thioester was not achieved, yet refolding into the histone octamer is not significantly affected by quantities of thioester present less than or equal to the amount of full-length H3-pT118 [Figure 10b]. This is because refolding into the histone octamer will only occur with full-length H3, and any remaining thioester may be removed when the octamer is purified.

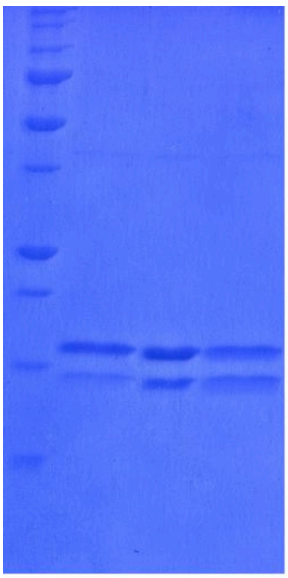
a.



b.

MW ladder

Purified H3-pT118



H3-pT118

H3(1-109)SR

Figure 10: Ion-exchange purification of H3-pT118

a. HPLC 10-60% B gradient (see appendix I) over 50 minutes. Note the full-length H3 cannot be completely separated from H3(1-109)
b. 15% SDS-PAGE of purified H3-pT118

IV: Refolding H3-pT118 into the histone octamer

H3-pT118 was refolded into the histone octamer by adding equimolar amounts of each histone protein under 7M guanidine denaturing conditions to a button [Figure 11a&b], and slowly dialyzing away the guanidine, forming the histone octamer through gradual refolding.

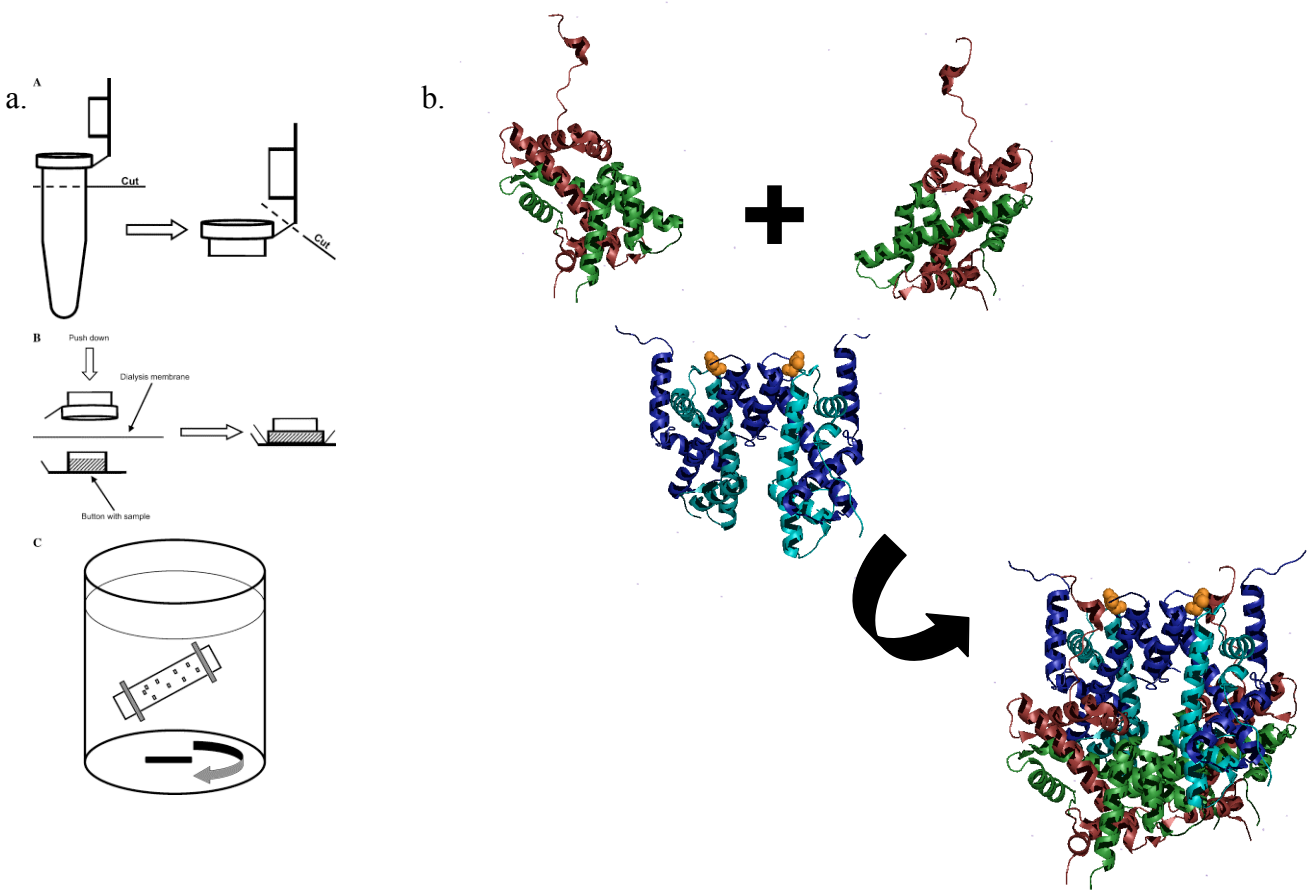


Figure 11: Refolding schematic

a. Assembly of buttons for double-dialysis
b. H2A, H2B, H3-pT118, and H4 are combined to form the histone octamer.

Once the octamer was refolded, it was purified via FPLC gel filtration. This removes aggregates, H3-H4 tetramer, and H2A-H2B dimer that also form in refolding, and also the remnant H3(1-109)SR. Pure fractions of octamer were pooled and concentrated. SDS-PAGE and MALDI-TOF confirmed purity and the presence of all four histone proteins [Figure 12].

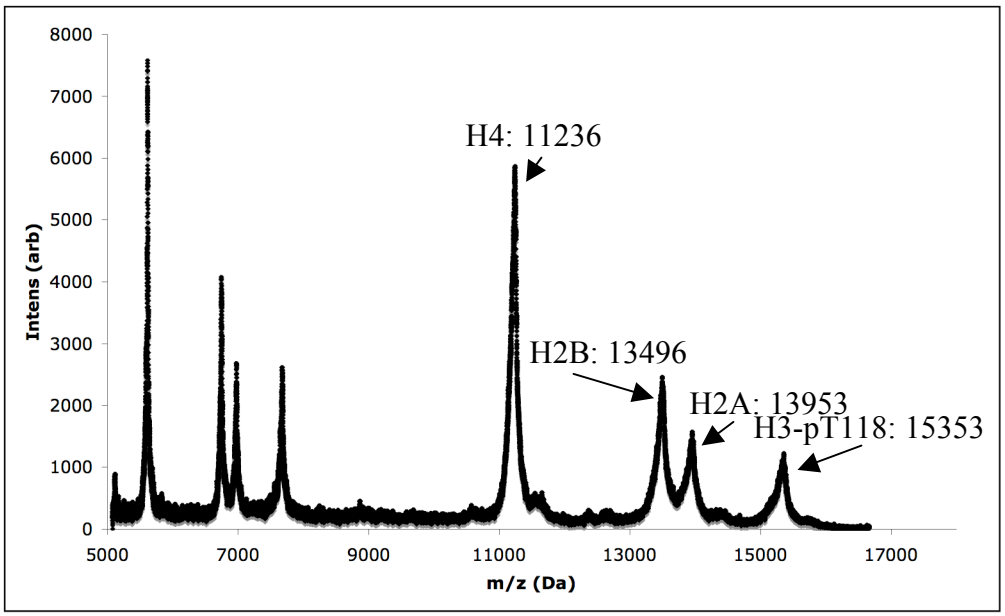


Figure 12: Purified octamer- MALDI-TOF confirms octamer purity (H2A expected m/z 13950, H2B expected m/z 13494, H3-pT118 expected m/z 15354, H4 expected m/z 11236)

V: Reconstitution into nucleosomes

Histone octamer containing H3-pT118 was reconstituted into nucleosomes with various DNA constructs containing the mp2 positioning sequence, a derivative of the 601 nucleosome positioning sequence [15],[16]. Construct mp2-147 simply contained the 147 bp of mp2. The mp2-187 construct contained the mp2 sequence flanked by 20 bp of nonpositioning DNA on each end, and mp2-247 contained of the mp2 sequence flanked by 50 bp of nonpositioning DNA on each end. The 5' end of each construct was labeled with Cy3, and the 3' end with Cy5, in order to enable imaging of the nucleosomes. Reconstitutions were performed with a 1:6 ratio of labeled to unlabeled DNA of the same sequence, in order to conserve labeled DNA. Reconstitution was accomplished by resuspending the histone octamer and DNA in 2M NaCl in a button in a baggie, and dialyzing against 0M NaCl while shielding against photobleaching of the fluorescent labels.

Once reconstituted, the products were run on a 5% acrylamide gel and imaged via fluorescence. Gel analysis reveals the presence of multiple species, including an anomalous species with decreased electrophoretic mobility compared to normal nucleosomes in the H3-pT118 reconstitutions, in addition to mononucleosomes in the mp2-147 and mp2-187 reconstitutions [Figure 13a&b].

Different anomalous structures appear on the mp2-247 construct, adding to the complexity of the system. The decreased electrophoretic mobile structures are still present in decreased amounts, but another structure, with intermediate electrophoretic mobility compared to the two mononuclesome species emerges in pT118 reconstitutions with mp2-247 DNA.

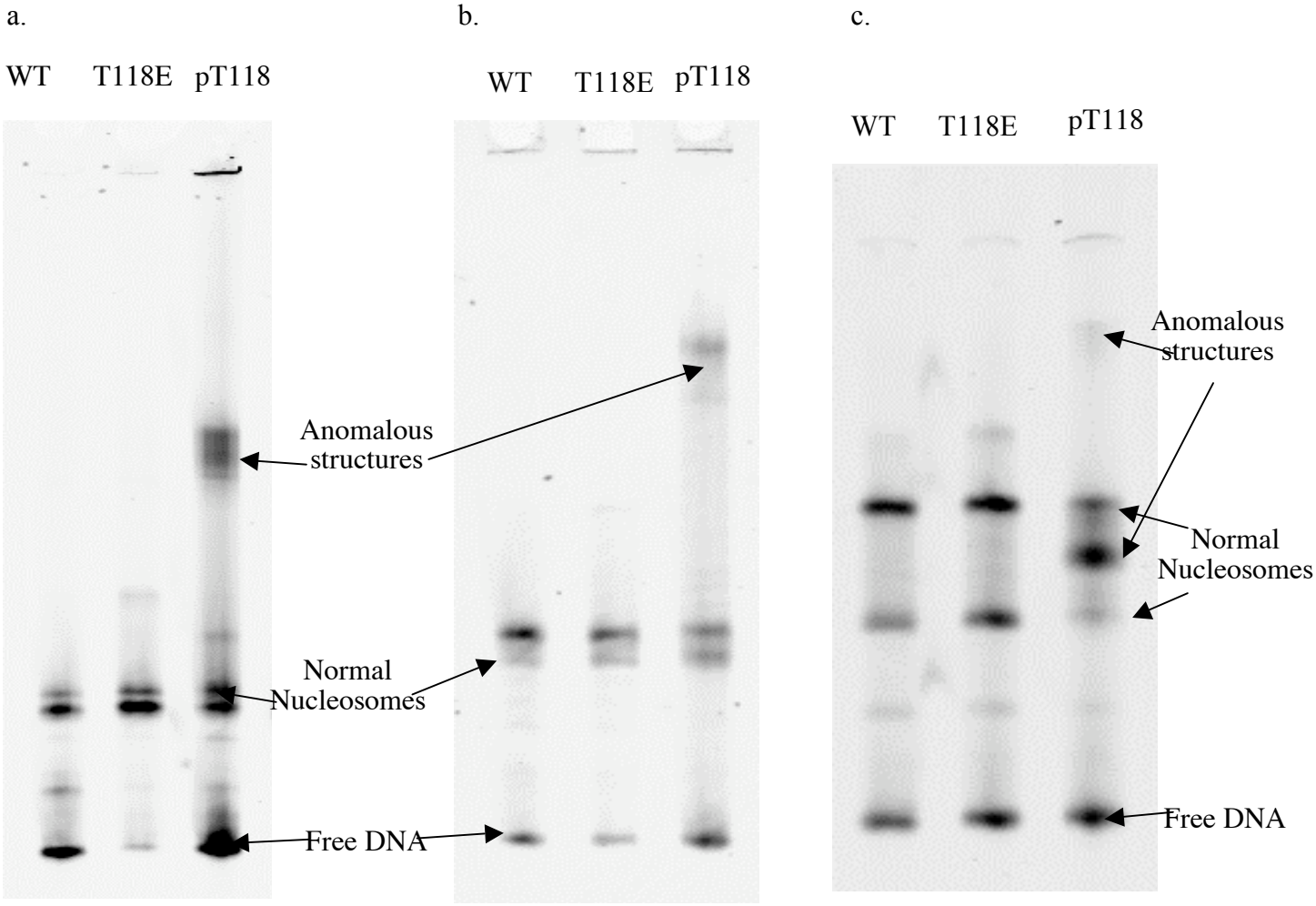


Figure 13: 5% native acrylamide gel of reconstitution products for unmodified (WT), T118E, and pT118

a. mp2-147 sequence
b. mp2-187 sequence
c. mp2-247 sequence

The reconstitution products were purified via sucrose gradient centrifugation in order to differentiate the two species present in pT118 reconstitutions, as well as to remove the free DNA still present. The gradient was fractionated and the presence of each species was confirmed by fluorescence and 5% native gel analysis [Figure 14]. Sucrose gradient sedimentation is supportive of the hypothesis that the anomalous structures are of a higher mass, as they sediment at a higher percentage of sucrose than normal nucleosomes. When the mp2-247 anomalous nucleosomes are purified, both the decreased and intermediate electrophoretic mobile species sediment at the same place in the gradient, similar to that of other constructs' anomalous structures.

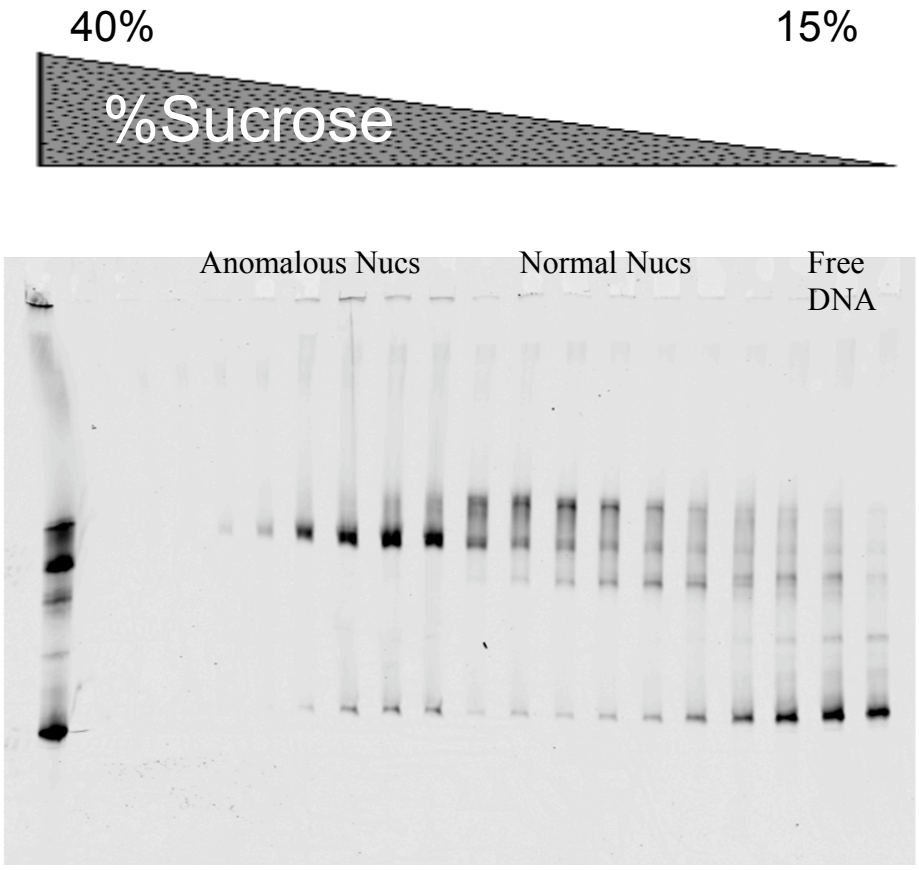


Figure 14: 5% native gel of sucrose gradient fractionation for mp2-247

Chapter 3: Analysis of H3-pT118 nucleosomes

I: CIP digestion of H3-pT118 octamer

Calf Intestinal Phosphatase was utilized in order to determine if the anomalous structures formed in reconstitution of H3-pT118 nucleosomes were due to the presence of the phosphate group or not. H3-pT118 octamer was treated with CIP in order to dephosphorylate the octamer, leaving only unmodified octamer. A negative control of unmodified octamer treated with CIP was also performed. Reconstitutions were done on the H3-pT118 + CIP octamer, H3-pT118 untreated octamer, unmodified + CIP octamer, and unmodified untreated octamer using DNA containing the mp2 sequence. The resultant products showed that the dephosphorylated H3-pT118 octamers did not form anomalous structures, instead forming identical structures to unmodified nucleosomes, as analyzed on a 5% native gel [Figure 15].

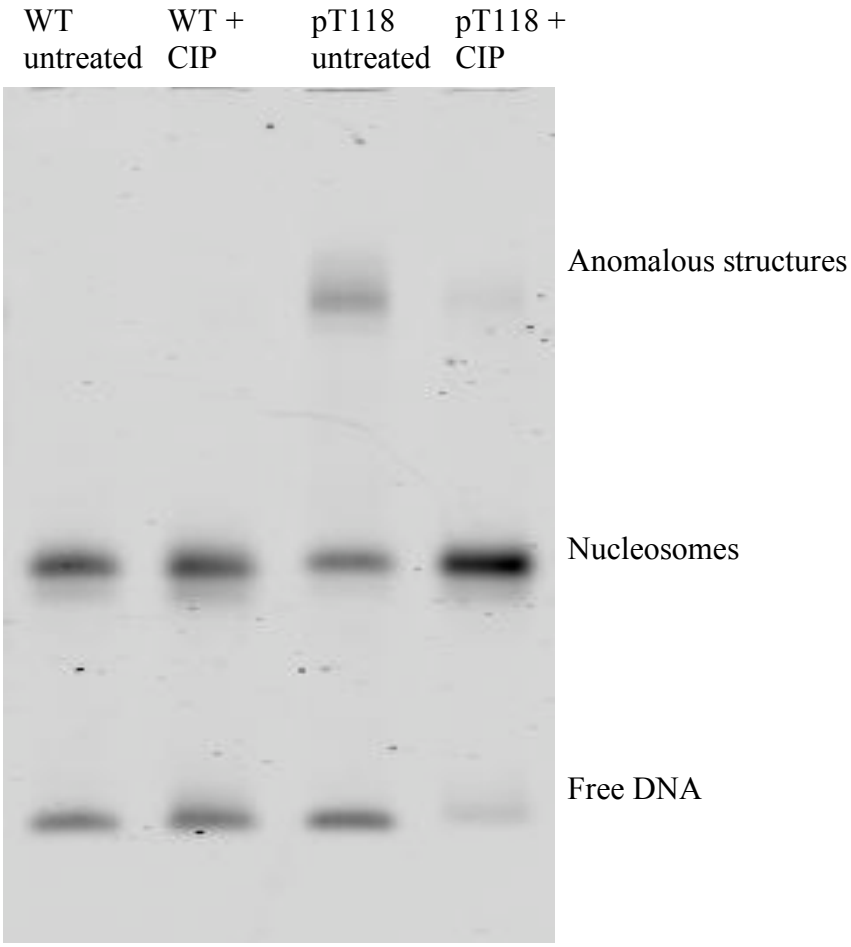


Figure 15: 5% native gel of the reconstitution of CIP treated WT and H3-pT118 octamers, as well as untreated WT and pT118 octamers

Preliminary MALDI-TOF studies suggest that dephosphorylation of only 50% of the octamer will lead to the formation of normal nucleosomes and the absence of the altered structures. However, successful reconstitution of CIP-treated octamer that has been analyzed via mass spectrometry remains to be performed.

II: ExoIII Mapping

Exonuclease III mapping was performed on H3-pT118 anomalous structures as well as H3-pT118 mononucleosomes to determine positioning of the octamer on the DNA sequence. ExoIII will digest DNA from the ends and will pause when it reaches the entry-exit region of the nucleosome, where the DNA first associates with the octamer. Because the structures are labeled with a different fluorescent label on each end, the length of DNA remaining from each end will tell where on the DNA the octamer is sitting relative to each end.

H3-pT118 purified anomalous structures and mononucleosomes, as well as unmodified nucleosomes and free DNA as controls were digested with ExoIII. The resultant timepoints were run on a dentaturing urea/acrylamide gel in order to determine the length of DNA pieces for each structure at each timepoint.

Interestingly, both the anomalous structures and mononucleosomes exhibit altered digestion, differing depending on the length of the DNA sequence. The anomalous structures on the mp2-247 sequence exhibit very little digestion, yet the digestion that does occur is in 10 bp increments with no pause site. This shows that the ends are protected in the mp2-247 anomalous structures for the most part. The mononucleosomes on the mp2-247 sequence do not exhibit the same character; rather, they exhibit significant digestion in 10 bp increments throughout the sequence [Figure 16]. This distribution of positioning is similar to that of thermally shifted unmodified nucleosomes, possibly indicating a role for H3-pT118 as a chromatin remodeler.

The anomalous structures on mp2-147 show a different distribution. Rather than barely digesting at all, they exhibit several pause sites in 10 bp increments throughout the sequence, indicating that the ends are not protected at all, and that there may be multiple structures present. The mp2-147 mononucleosomes also contain several pause sites in 10 bp increments that are not

present in unmodified nucleosomes, as well as a strong pause site at 50 bp in from the ends [Figure 17]. This indicates the mononucleosomes are depositioned from the center.

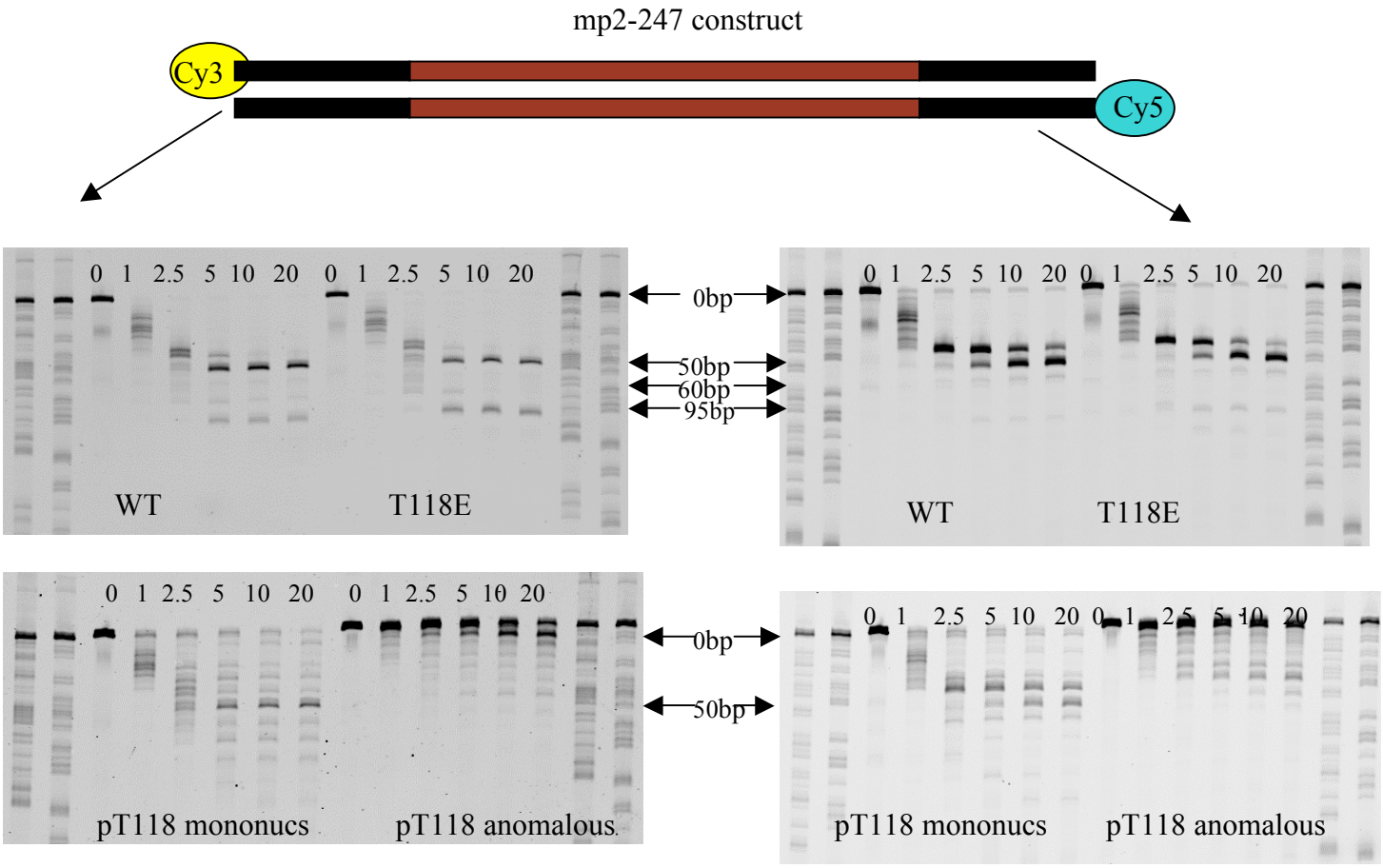


Figure 16: ExoIII mapping of mp2-247 H3-pT118 nucleosomes over 20 minutes.

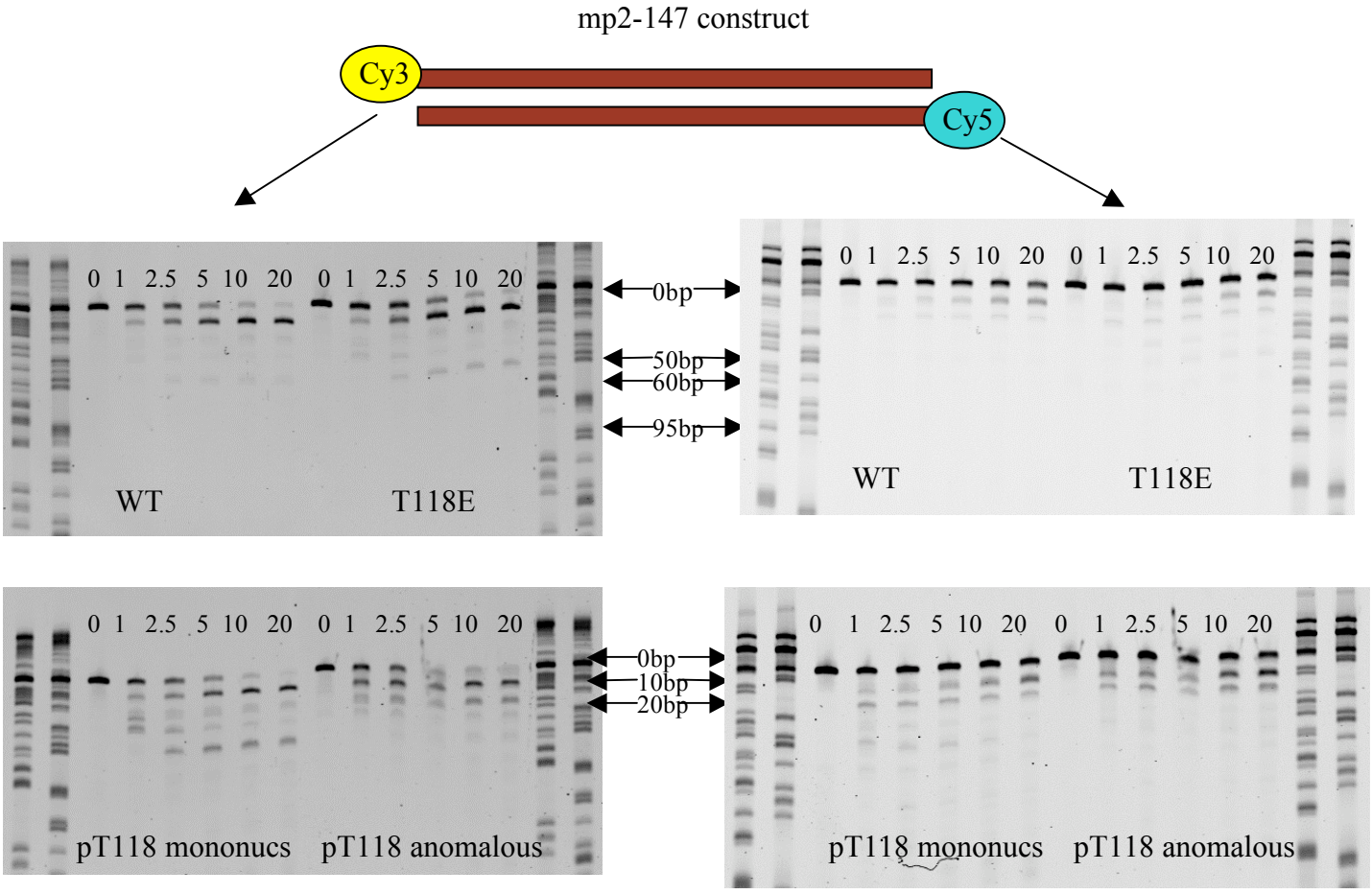


Figure 17: ExoIII mapping of mp2-147 H3-pT118 nucleosomes

III: DNAse Mapping

DNAse I mapping was performed on H3-pT118 anomalous structures, as well as on H3-pT118 mononucleosomes in order to determine the accessibility of DNA on the modified histone octamer in the two structures. DNAse I will cut the DNA at any point in the sequence at which is exposed and the enzyme may access. On an unmodified nucleosome, only the ends are accessible, as the rest of the DNA is tightly bound to the histone octamer and thus inaccessible. The Cy3 and Cy5 labelled ends also tell how far from each end cleavage sites are.

Further details into the structure of anomalous nucleosomes were gained from the DNAse I digestions. Like unmodified nucleosomes, H3-pT118 mononucleosomes exhibit defined regions of protection of the DNA, especially at the dyad region. However, the anomalous structures show decreased digestion overall, yet no particular region of the DNA seems to be protected [Figure 18]. All 3 DNA constructs showed the same pattern for both structures. This may indicate that the anomalous structures are not necessarily one particular structure, and are a heterogeneous mixture.

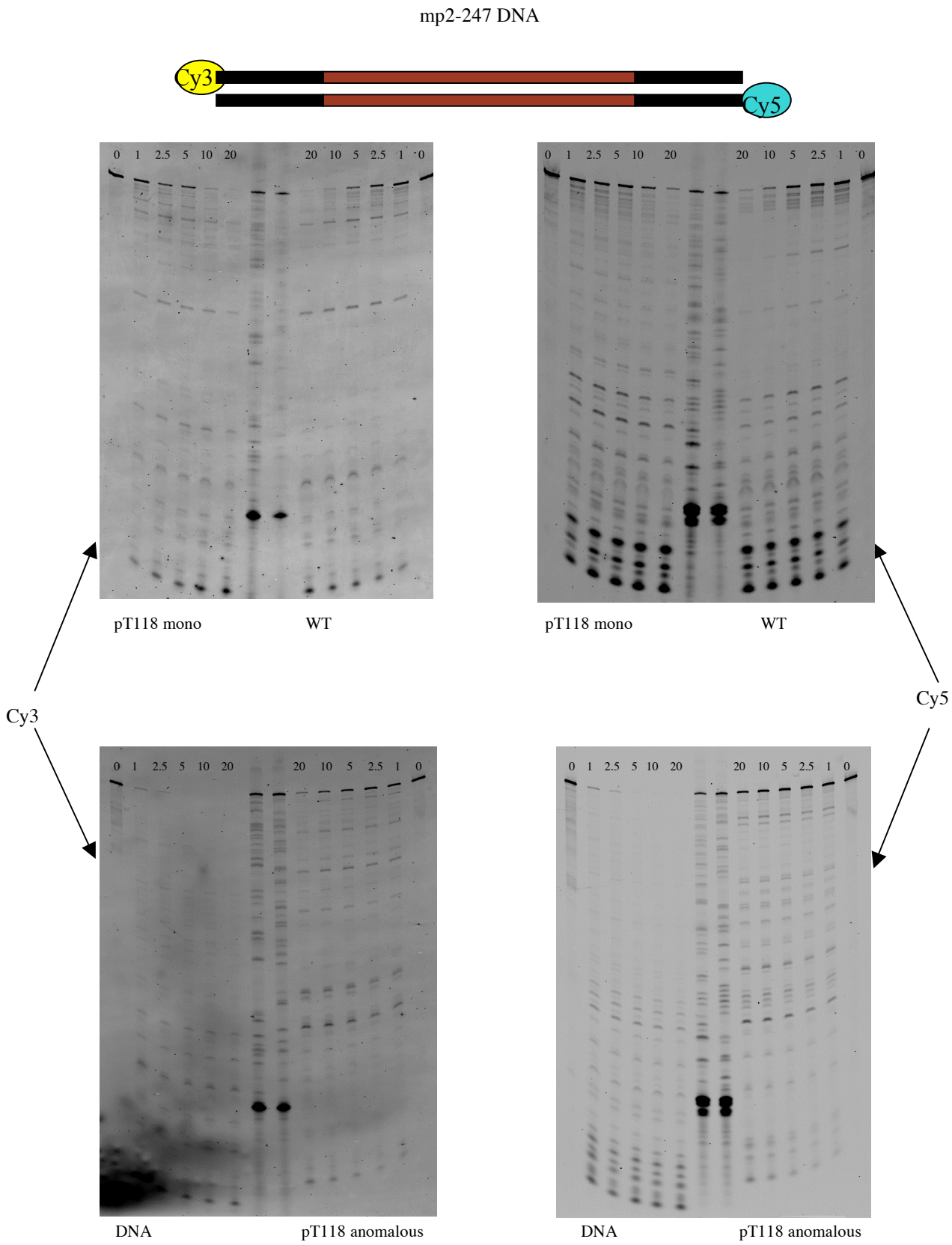


Figure 18: DNAse mapping of mp2-247 H3-pT118 nucleosomes over 20 minutes

V: Atomic Force Microscopy Imaging

H3-pT118 anomalous structures and mononucleosomes, as well as unmodified nucleosomes prepared in our lab were sent to Dr. John Van Noort (at Leiden University) to be imaged via atomic force microscopy. Atomic force microscopy provides a direct view of the 3D shape and size of molecules, in this case, fixed in place.

The images show the H3-pT118 anomalous structures are apparently larger in size than unmodified nucleosomes [Figure 19]. Degradation of the anomalous structures appears to have occurred either in transit or in the fixing process, as free DNA is clearly visible in the field as well. Nevertheless, confirmation that the anomalous structures are larger in size confirms what was suspected due to electrophoretic mobility and sucrose gradient sedimentation. H3-pT118 mononucleosomes appear to be similar in structure to unmodified nucleosomes, indicating that they are likely similar structures to unmodified nucleosomes.

WT nucleosomes pT118 anomalous

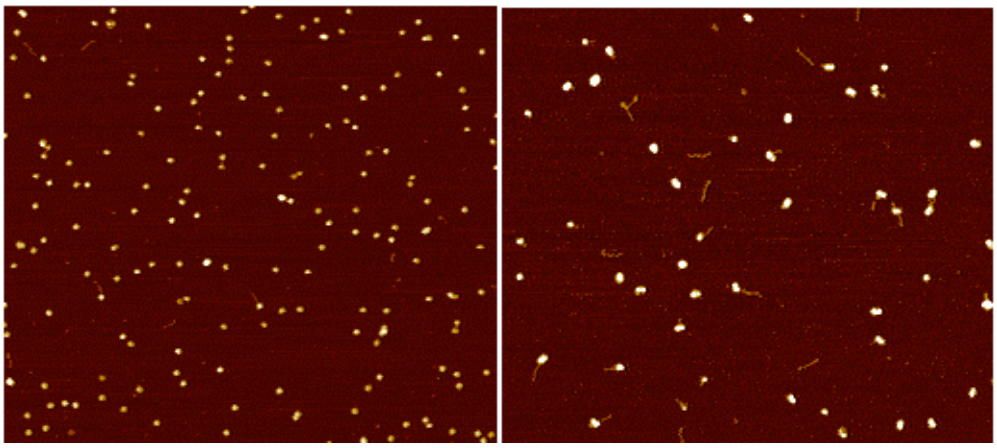


Figure 19: Zoomed in (to scale) AFM images of WT, H3-pT118 mp2-147 anomalous structures

VI: Protein Content Determination

In order to determine the relative amount of each histone protein, H2A, H2B, H3, and H4 in the anomalous H3-pT118 structures, an SDS-PAGE gel was run to visualize the ratios between the 4 proteins. As H2A and H2B are approximately the same weight (13.5 kDa), H3 is approximately 15 kDa, and H4 is approximately 11.5 kDa, unmodified nucleosomes made with full octamers will show a ratio of 1:2:1 H3: H2A/H2B: H4. The H3-pT118 anomalous structures also show this ratio, with multiple different DNA sequences [Figure 20], as quantified by ImageQuant software. This suggests that H3-pT118 anomalous structures are composed of full octamers of histone proteins paired with DNA. What remains to be determined is the number of octamers per segment of DNA in each anomalous structure.

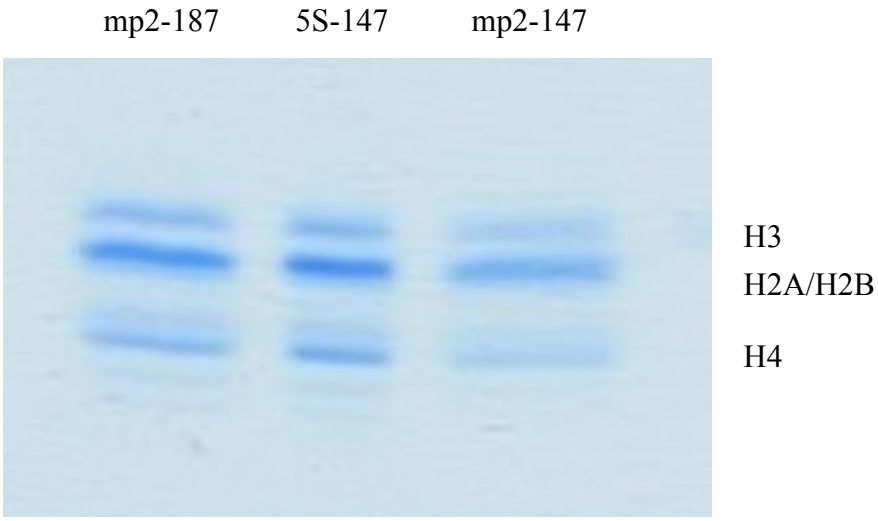


Figure 20: 15% SDS-PAGE gel of H3-pT118 ANs

Chapter 4: Discussion

Phosphorylation of H3-T118 has the potential to dramatically alter chromatin structure, due to the close proximity of the residue to the DNA phosphate backbone [6]. We have shown that this modification does indeed lead to the formation of anomalous structures when H3-pT118 modified octamers are reconstituted with short segments of DNA. The physiological significance of this modification may lie in the ability to form these higher-order structures beyond mononucleosomes, as they are similar to higher-order structures formed when unmodified normal nucleosomes are treated with chromatin remodeling enzymes, such as SWI/SNF [17]. This is further complicated, due to the fact that mutations at H3-T118 have been shown to relieve the need for chromatin remodeling enzymes for gene transcription in yeast [8]. This could indicate that phosphorylation of H3-T118 is a chromatin remodeling modification.

The ways in which H3-pT118 may remodel chromatin are not quite clear, however. The ExoIII data on H3-pT118 mononucleosomes with longer stretches of DNA indicate increased accessibility of the ends of DNA in single nucleosomes, indicating depositioning. One manner in which chromatin remodeling is believed to occur is by sliding nucleosomes along DNA, effectively depositioning them [18]. The fact that H3-pT118 mononucleosomes deposition without the application of external heat further supports the role for H3-pT118 as a chromatin remodeling modification.

The anomalous structures formed by H3-pT118 reconstitutions are interesting as well, as they may be a clue as to what kind of structures actually form when chromatin is remodeled. ExoIII mapping of the anomalous structures reveals that ends may or may not be protected in the anomalous structures, although decreased digestion overall indicates the ends are usually protected when short DNA segments are utilized. Furthermore, DNAse I digestion indicates a

similar story. There is no particular area within the short DNA sequences which seems to be protected, rather, there is decreased accessibility throughout the entire sequence. This may indicate that the anomalous structures are not nucleosomal, such as a simple dinucleosome, rather they are composed of DNA wrapped around the histone octamer in novel ways.

The composition of the anomalous structures is also in question. The protein content gels indicate that they contain the normal ratio of histone proteins, suggesting that they are composed of full histone octamers associated with DNA. Also, they run similar to dinucleosomes on a native acrylamide gel and sediment similarly on a sucrose gradient indicating that they may be composed of two octamers and two pieces of DNA. However, mp2-247 reconstitutions exhibit two unique anomalous structures. The first is similar to the mp2-147 and mp2-187 structures in electrophoretic mobility and sucrose gradient sedimentation, similar to dinucleosomes. However, the second has increased electrophoretic mobility when compared to the first anomalous structure. The electrophoretic mobility is in between those of the two different mononucleosome positionings, yet sucrose gradient sedimentation is similar to that of the first anomalous structure. This particular structure may be composed of 2 octamers on 1 piece of DNA, as the DNA segment utilized is longer, and may indeed wrap around 2 octamers to form a similar electrophoretic mobility and density as observed. However, in order to determine the composition of these structures, further work is required.

One way in which we look to probe the composition of the different anomalous structures is by labeling the histone octamer on H2A with a Cy5-labeled cysteine, and reconstituting with DNA labeled with Cy3 at various positions. The resultant products may then be imaged using fluorescent imaging at both Cy3 and Cy5, in order to determine the ratio between the two signals. A control will be done in which the ratio of octamer to DNA will be measured using

Cy5 labeled unmodified octamer and the same Cy3 labeled DNA. This will allow us to more definitively determine the composition of anomalous structures.

In addition to determining the composition of the anomalous structures formed with short segments of positioning DNA, different lengths of DNA may be reconstituted with H3-pT118 octamer to determine the effects of the modification on longer-range structure. This will determine whether the modification is only capable of forming dinucleosome-like anomalous structures, or whether larger aggregates of octamer and DNA are formed on longer sequences. This will be done by reconstituting H3-pT118 octamer onto nucleosome arrays of DNA segments with > 2 nucleosomes worth of DNA. DNAse digestion of these arrays will help determine the arrangement of DNA on the octamers, while AFM images may give us clues as to the higher order structure.

Appendix I: Experimental Methods

Fmoc synthesis of H3(110-135)-pT118: Synthesis of H3-pT118(110-135) was performed on a 0.125mmol scale, starting with 500mg of Fmoc preloaded Ala-Wang resin. The resin was deprotected using 20% piperidine. Each amino acid was activated by adding .5mmol HBTU and 0.25mL DIEA. The activated amino acid was then coupled onto the deprotected resin and N_2 was bubbled through for 45 minutes. After flow washing, a ninhydrin test was performed in order to determine the completion of the reaction, indicated by an absence of free amines. Couplings that did not proceed to completion were double coupled and capped with acetic anhydride. A test cleavage with TFA was performed on a small portion after the addition of H3-M120 to ensure the correct product was present. Before coupling V117, a wash with 10% DIEA was performed to prevent pT118 from binding piperidine. After completion of the full peptide, test cleavages were done with 2,4,6 hour timepoints in 94.0% TFA, 2.5% H_2O, 2.5% EDT, and 1% TIS, as well as with a 4 hour timepoint in Reagent K, 82.5% TFA, 5% phenol, 5% H_2O, 5% thioanisole, and 2.5% ethanedithiol. Large scale cleavage was performed in Reagent K. The TFA mixture was evaporated off, and the peptide was precipitated with 3 ether washes. Analytical RP-HPLC and MALDI-TOF mass spectrometry determined the presence of the correct peptide. The N-terminal thioproline ring was opened, deprotecting the cysteine by reaction with 0.4M methoxylamine in 30% acetonitrile at pH 4 for 4 hours, followed by lyophilization.

HPLC Purification of H3(110-135)-pT118: RP-HPLC purification conditions were determined on an analytical scale before proceeding to prep scale purification. The desired peptide was resuspended in 30% acetonitrile and run on a Grace-Vidac C18 column on a 0-73% 30 minute

Buffer B gradient (90% Acetonitrile, 10% H2O, and 0.1% TFA). The desired peptide eluted around 25% B, so gradients of 15-45% and 15-35% over 30 minutes was determined to increase resolution between peaks. Large-scale purification of the peptide was performed on a Grace-Vidac C18 column on various gradients with 30mg crude per injection. Individual fractions were collected and samples were run on the analytical C18 column to determine purity. Each fraction was also analyzed via MALDI-TOF to confirm purity. Fractions with >5% purity were combined, and purity was confirmed by RP-HPLC and MALDI-TOF.

Expression of H3(1-109)SR: The sequence for H3(1-109)SR was cloned into pTXB1 (New England Biolabs) vector and transformed into BL21 cells. Glycerol stocks of the BL21 cells were made and used for each subsequent cell expression. 6 5mL overnight cell cultures were grown from the glycerol stock in LB broth containing 0.1mg/mL Ampicillin (Amp) at 37°C with shaking. After 16 hours, each overnight was added to 500mL of LB broth with 0.1mg/mL Amp at 37°C with shaking. The OD(600nm) of each flask of the cells were measured until OD(600nm) was greater than 0.400. Once the OD was high enough, the cells were induced by adding IPTG to a final concentration of 0.2mM in each flask. Expression of the protein was optimized by growth for 2.5 hours at 37°C with shaking. Induced and uninduced cell pellets were run on a 12% SDS-PAGE gel to confirm expression. The cells were centrifuged at 5000 rpm for 20 minutes at 4°C, and the supernatant fluid was removed. The pellets were then resuspended in 1M NaCl, 25mM HEPES pH 7.5, and 1mM EDTA, with 1 mM PMSF. The resuspended cells were stored at -80°C and then lysed with a French press. The lysate was centrifuged at 15,000rpm for 20 minutes at 4°C, and the supernatant was removed. The pellet was washed twice with 1% triton, 25mM Hepes pH 7.5, 1M NaCl, 1mM EDTA. The cell pellet

was soaked with 250 uL of DMSO, then mixed in 1M NaCl, 25mM HEPES pH 7.5, 1mM EDTA, and 5M urea. The mixture was centrifuged at 15,000rpm for 10 minutes at 4°C, and the supernatant was saved. The pellets were washed with 1M NaCl, 25mM HEPES pH 7.5, 1mM EDTA, and 6M urea and centrifuged again, saving the supernatant. The supernatant was diluted to 300mM NaCl and run on a GE Healthcare SP-FF ion exchange column. One wash with 50mL 0M NaCl buffer was performed, and then the protein was eluted with 1M NaCl buffer. The flow thorough, wash, and each fraction were run on a 15% SDS-PAGE gel. The fractions containing protein were concentrated in an Amicon-50 15,000 MWCO to <7mL. The concentrated protein solution was then run on a sepharacyl S200 gel filtration column in 20mM sodium acetate, 1M NaCl, 1mM EDTA, and 7M urea. Fractions were analyzed via 15% SDS-PAGE and pure fractions were pooled and dialyzed overnight at 4°C against 1M NaCl, 25mM HEPES pH 7.5, and 1mM EDTA to remove the urea. MESNA was then added to the mixture to 50 mM, and cleavage of H3(1-109)SR from the intein-CBD proceeded for 22 hours at 4°C. The solution was adjusted to 6M urea, and concentrated in a Centricon 5000 MWCO to >1mg/mL H3(1-109)SR concentration. The protein was stored at -80C prior to ligation.

Ligation to H3(1-109)SR: 10 mM TCEP and 50 mM MESNA were added to the thioester solution. H3(110-135)-pT118 peptide was added to 2.5 times greater concentration than thioester by mass. The reaction was allowed to nutate at room temperature for 16 hours. The peptide and urea were dialyzed away against 5mM BME over 2 buffer changes, and the remaining protein was lyophilized.

Ion exhange purification of H3-pT118: The ligation products were resuspended in 7M urea, 10mM Tris pH 9, 1mM EDTA, 5mM BME, and 100mM NaCl. The protein concentration was determined via UV spectroscopy, $\varepsilon = 4040\ M^{-1}cm^{-1}$. It was then loaded on an ion exchange column and run on a 10-60% buffer B gradient (10mM Tris pH 9, 1mM EDTA, 5mM BME, and 1000mM NaCl) over 50 minutes. Fractions were collected and the identity of peaks and purity were determined by running each fraction on a 15% SDS-PAGE gel. Fractions with >50% full-length H3-pT118 compared to H3(1-109)SR were pooled and dialyzed against 5mM BME. The pure protein was dried by speedvac and stored at -80°C prior to refolding.

Refolding of H3-pT118 into the histone octamer: Purified H3-pT118, as well as H2A, H2B, and H4 were resuspended in unfolding buffer: 7M Guanidine, 20 mM Tris pH 7.5, 10 mM DTT, and the concentration was determined via UV-Vis spectroscopy. Each histone was added in an equimolar ratios and the volume was adjusted with unfolding buffer to 50 μL total volume. The histone mixture was assembled in a button (the cap removed from a 0.65mL microcentrifuge tube assembled with 6-8000 MWCO dialysis membrane holding in the reaction mixture) and the button was placed in a 6-8000 MWCO dialysis tube filled with unfolding buffer. This was then placed in a 4L bucket with refolding buffer, 2M NaCl, 1mM EDTA, and 10mM Tris pH 7.5 at 4°C. The buffer was changed twice, and the samples were reclaimed and stored on ice.

FPLC Purification of H3-pT118 histone octamer: The octamer was purified via FPLC gel filtration on a superdex 200 column in refolding buffer. The major products of the reaction were H2A/H2B dimer, H3/H4 tetramer, and octamer containing H3(1-109)SR instead of full length

H3-pT118. Fractions were collected and run on 15% SDS-PAGE gels to determine purity. Pure fractions were pooled and concentrated to a concentration of >2 mg/mL.

Reconstitution into nucleosomes: Octamer and DNA were reconstituted into nucleosomes by assembling the following reaction mixture in a button: 2M NaCl, 0.5X TE, 1mM BZA, equimolar HO and DNA, and H2O so that the total volume is 50 μL. The button was placed in a baggie of 6-8000 MWCO dialysis tubing of 2M NaCl, 0.5X TE, and 1mM BZA, and dialyzed against 4L of 0.5X TE and 1mM BZA at 4°C under dark conditions. The buffer was changed after 8 and 16 hours, and after 32 hours the samples were reclaimed. To determine the electrophoretic mobility of the products, the sample was run on a 5% native acrylamide gel at 300V for 1 hour and imaged via fluorescence imaging. The DNA sequences used were mp2-147, mp2-187, and mp2-247, which were labeled with Cy3 at the 5' end and Cy5 at the 3' end, enabling imaging.

Sucrose Gradient purification: Nucleosomes and anomalous structures were isolated from free DNA and each other by sucrose gradient centrifugation. 5-40% sucrose gradients were poured and reconstitution products were loaded. The gradients were centrifuged at 41,000rpm at 4°C for 18 hours, and then fractionated. The individual fractions were analyzed by fluorescence and by running on a 5% native gel to determine purity of each fraction. Pure fractions were pooled as either anomalous structures or mononucleosomes.

Dephosphorylation: Dephosphorylation of H3(110-135)-pT118 was optimized prior to reaction with octamer to ensure the protocol was working. The following reactions were attempted: 1 μg

peptide in 1X NEB buffer 3, 10 U CIP, 0.5M NaCl in 12 μL total volume, the same reaction with half the CIP, and the same reactions in 5 μL total volume. After 60 minutes, all reactions had proceeded to >95% dephosphorylation as observed by MALDI-TOF. The 1 ug CIP, 12 μL reaction was attempted with 1 μg of H3-pT118, and also with 1 μg of H3-pT118 octamer. MALDI-TOF analysis confirmed significantly lower dephosphorylation rates after desalting the sample with Millipore ZipTip C18 treatment. Previous reconstitutions of CIP treated H3-pT118 octamer yielded no formation of anomalous structures by 5% native gel analysis.

ExoIII mapping: pT118 mononucleosomes, pT118 anomalous structures, WT nucleosomes, and free DNA were digested with ExoIII at 166U/mL concentration. The reaction was carried out in 0.5mM $MgCl_2$ and 20mM Tris pH 8 at 16°C, with a total volume of 25 μL per reaction. The ExoIII stock was prepared separately from the nucleosome reaction mixture, and incubated at 16°C for 3 minutes before adding the ExoIII to the nucleosome mixture. Timepoints were taken at 0, 1, 2.5, 5, 10, and 20 minutes by quenching 7 μL of the reaction with 1 μL of 0.16M EDTA. Samples were prepared for a denaturing gel by digesting with 1 μg of Proteinase K and 1 μL of 0.2% SDS per timepoint for 20 minutes at 37°C. The samples were then treated with 10 μL of formamide and heated to 95°C until the gel was loaded. The samples were run on an 8% acrylamide, 7M urea gel in 1X TBE until a dye lane ran off the gel. The gels were imaged by fluorescence at Cy3 and Cy5.

DNAse mapping: DNAse mapping was done under the same conditions as ExoIII, with the exception that the concentration of DNAse in each reaction had to be determined with each individual batch of DNAseI. A titration of 0, 10, 12.5, 16, 20, 25, 32, and 40 U/mL final

concentrations of DNAse I were attempted on a constant 1 μg of DNA per reaction in order to determine the optimal digestion concentration. Then, that concentration was used for the pT118 mono, pT118 AN, and WT digestions. Timepoints were taken at 0, 1, 2.5, 5, 10, and 20 minutes and the samples were treated and run on a denaturing gel in the same manner as the ExoIII digestions.

Appendix II: Acronym Table

DNA- Deoxyribonucleic Acid
Thz- Thiazolidine
CBD- Chitin-Binding Domain
HBTU- O-Benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate
DIEA- Diisopropylethylamine
TFA- Trifluoroacetic Acid
EDT- Ethanedithiol
TIS- Triisopropyl Silane
RP-HPLC- Reverse Phase High Pressure Liquid Chromatography
MALDI-TOF- Matrix Assisted Laser Desorption/Ionization Time Of Flight
TCEP- Tris(2-carboxyethyl)phosphine
LB- Luria-Bertani (broth)
SDS- Sodium Dodecyl Sulfate
SDS-PAGE- SDS-Polyacrilamide Gel Electrophoresis
HEPES- 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
EDTA- Ethylenediamine Tetraacetate
PMSF- Phenylmethanesulphonylfluoride
MWCO- Molecular Weight Cut Off
MESNA- Sodium 2-sulfanylethanesulfonate
BME- Beta-Mercaptoethanol
TE- Tris-EDTA buffer (1x = 10 mM Tris pH 7.5, 1 mM EDTA)
BZA- Benzamidine
CIP- Calf Intestinal Phosphatase
TBE- Tris-Borate-EDTA buffer (1x = 100 mM Tris pH 7.5, 100 mM borate, 2 mM EDTA)

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